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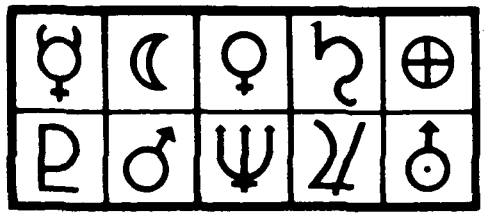

PLANETARY QUARANTINE

QR 22
SEPTEMBER 1971

SANDIA LABORATORIES QUARTERLY REPORT PLANETARY QUARANTINE PROGRAM

Prepared by:
Planetary Quarantine Department 1740

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SANDIA LABORATORIES QUARTERLY REPORT - PLANETARY QUARANTINE PROGRAM

TWENTY-SECOND QUARTERLY REPORT OF PROGRESS

For

Period Ending September 30, 1971

Planetary Quarantine Department
Sandia Laboratories, Albuquerque, New Mexico

Project No. 0064010

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SANDIA LABORATORIES QUARTERLY REPORT
PLANETARY QUARANTINE PROGRAM

Summary of Activities

Planetary Quarantine Analysis. Two studies were undertaken this past quarter. First, a quantitative means was developed to investigate the sensitivity of current spacecraft sterilization plans to variations in D-values. The results show that the success of current plans is easily affected by the existence of a few organisms whose D-value is more than twice that required by the current sterilization plans. The second study represents an attempt to derive a quantitative expression representing the distribution of D-values among a population of naturally occurring organisms. A rationale was developed which suggested that inactivation rate may be log normally distributed. This distribution was then compared with two others that seemed qualitatively reasonable. The log normal gave a far superior representation of the distribution in inactivation rate when compared with data than did the others.

Thermoradiation Experimentation. Activities this quarter have been devoted primarily to the investigation of (1) the inactivation of both Bacillus subtilis var. niger spores and Cape Kennedy soil spores by γ-radiation at room temperature in a nitrogen environment and (2) the thermoradiation resistance of Cape Kennedy soil spores at elevated temperatures below 125°C. Some tentative conclusions are:

- Radiation inactivation rates (in both cases) are increased in a nitrogen environment, in agreement with other findings.

- The degree of sensitivity of inactivation rates to nitrogen (or lack of oxygen) appears to depend upon dose rate.
- There is almost no difference between the rate of inactivation of Cape Kennedy soil spores at 125°C and at 120°C (at 57 krads/hr) suggesting that thermoradiation inactivation of hardy spores may be accomplished efficiently at temperatures below 125°C.

Study to Evaluate Possible Clumping Effects of Cape Soil on Highly Heat Resistant Spores. There has long been a question about how realistically standard survival experiments with bacterial spores in soil represent results that would be obtained on spacecraft surfaces. To attempt to answer this question, spores in Cape Kennedy soil were placed on surfaces so that the spore density was no greater than 40 spores per square foot. The surfaces were microscopically observed, and no evidence of "clumping" found. This situation was felt to more fairly represent the actual configuration of spores and soil on spacecraft surfaces. Heat survival characteristics were subsequently studied under these conditions, and the results indicate that the resistance of Cape Kennedy soil spores is not appreciably different when "unclumped" from that encountered in standard survival experiments.

Studies on Bacterial Spore Inactivation. Both sporostatic and sporocidal properties may be ascribed to aqueous formaldehyde, as reported last quarter. This quarter, it was found that the sporocidal properties can be considerably increased by elevating the temperature - representing, in effect, a synergistic inactivation using combinations of aqueous formaldehyde and heat. A possible chemical explanation of this phenomenon is given.

Federal Standard 209a. Sandia Laboratories, through the AEC, has been authorized by the General Services Administration to revise Federal Standard 209a, "Clean Room and Work Station Requirements, Controlled Environment." As an initial step, letters were sent to the DOD, Army, Navy, Air Force, AEC, and NASA requesting appointment of agency representatives for this project.

Planetary Quarantine Analyses

A. Description. This is a continuing activity undertaken on an as needed basis which is devoted to identifying planetary quarantine problems and determining ways in which such problems may be approached and resolved with minimal effect on planetary exploration.

In our previous quarterly report (QR-21, June 1971) it was proposed that sterilization plans should be based upon the assumption of a mixture of D-values for a spacecraft bioburden rather than upon the assumption of a "maximum" D-value for the total spacecraft bacterial population. It was pointed out that this approach permitted achievement of planetary quarantine objectives with the minimum sterilization time.

B. Progress. Two substudies in this area were undertaken this quarter. The first (described in 1., below) determined the sensitivity of current sterilization plans to variation in D-values, and the second represents an attempt to define the nature of variation in D-values of organisms on a spacecraft in a quantitative fashion (2., below).

1. Sensitivity of Current Plans to Variations in D-Value

Given that there exist naturally occurring organisms with D-values well in excess of those currently specified for spacecraft sterilization, one may reasonably inquire how sensitive planetary quarantine requirements are to the presence of such organisms using currently prescribed approaches to spacecraft sterilization.

The following approach was taken to determine the sensitivity of current plans to variations in D-value. It was supposed that there were some N(0) surface (or mated surface or encapsulated) organisms present on the spacecraft surface. Knowledge of N(0), the planned D-value, D, (30 minutes for surfaces, etc.) and the level to which the population must be reduced (denoted ϵ) determines the planned sterilization time, t_s. In the general case t_s is the largest time (minutes) for which the inequality

$$N(0) \cdot 10^{-t/D} \leq \epsilon \qquad (1)$$

is satisfied.

If it is imagined that the N(0) organisms are, in reality, composed of two subpopulations containing $N_1(0)$ and $N_2(0)$ organisms, the first not "hardy" and the second "hardy," it may then be determined what part of N(0) that $N_2(0)$ may be and still have equation (1) satisfied. This is illustrated in Figure 1. The planned survivor curve (representing equation (1)) is shown. This is compared with a population the majority of which is inactivated rapidly (D-value, $D_1 = \frac{1}{2} D$) and a small part of which is inactivated slowly (D-value, $D_2 = 3D$). In Figure 1, N(0) is imagined to be 10^5 and ϵ is chosen to be 10^{-2}.

From Figure 1, one may deduce that under these circumstances, if the number of hardy organisms $N_2(0)$ exceeds about 2, the sterilization cycle is inadequate.

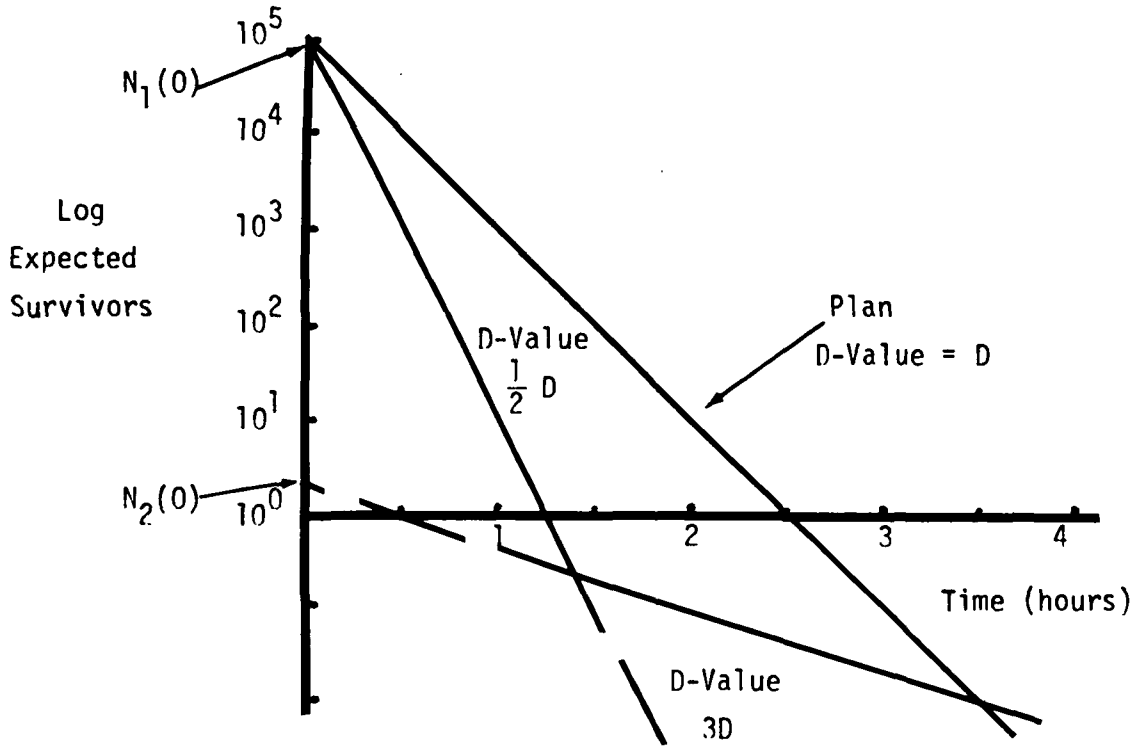


Figure 1 - Two Hypothetical Subpopulations Compared With Plan

More generally, it may be shown that an upper bound on the number, $N_2(0)$, of "hardy" organisms in a population of N(0) organisms is given by

$$N_2(0) \leq N(0) \cdot \frac{\left\{ \frac{\varepsilon}{N(0)} - \frac{\varepsilon^{1/\alpha_1}}{[N(0)]^{1/\alpha_1}} \right\}}{\left\{ \frac{\varepsilon^{1/\alpha_2}}{[N(0)]^{1/\alpha_2}} - \frac{\varepsilon^{1/\alpha_1}}{[N(0)]^{1/\alpha_1}} \right\}} . \qquad (2)$$

Here, the $N_2(0)$ organisms have a D-value $\alpha_2 D$ ($\alpha_2 \geq 1$) and the remaining $N(0) - N_2(0)$ organisms have a D-value $\alpha_1 D$ ($\alpha_1 \leq 1$) while the sterilization plan assumes all $N(0)$ organisms have a D-value, D.

Two comments are appropriate. The expression given in (2) is independent of the actual planned D-value, D, and is thus equally applicable to surface, mated surface or encapsulated organisms. Secondly, equation (2) is valid independent of the sterilization temperature profile.

Typical results that may be obtained from equation (2) are shown in Table I. One may see from this table, for example, that if the initial population is 10^5, the D-value for the $N_2(0)$ hardy organisms is 3D ($\alpha_2 = 3$, and D is the D-value for the sterilization plan), 10^{-2} is the terminal population requirement, and the remaining population $N_1(0) = N(0) - N_2(0)$ has a D-value of $\frac{1}{2}$ D, then $N_2(0)$ may not exceed 2 if the planned sterilization cycle is to be adequate. This was seen in Figure 1 earlier. Notice from Table I that if <u>any</u> organisms which have D-values no less than 3.5D are present in a population of 10^5, then the sterilization plan will be inadequate. A general perusal of Table I will convince one that current sterilization plans can be extremely sensitive to variations in D-value.

TABLE I - Upper bounds for the number of "hardy" organisms with D-value $\alpha_2 D$, for a sterilization cycle based on a D-value of D, terminal expected population of 10^{-2} and $\alpha_1 = 1/2$.

Hardiness Factor, α_2, for $N_2(0)$ Organisms	Total Population $N(0) = N_1(0) + N_2(0)$						
	10	10^2	10^3	10^4	10^5	10^6	10^7
1.5	0	4	21	99	464	2154	10,000
2.0	0	0	3	9	31	100	316
2.5	0	0	0	2	6	15	39
3.0	0	0	0	0	2	4	10
3.5	0	0	0	0	0	1	3
4.0	0	0	0	0	0	0	1
5 or greater	0	0	0	0	0	0	0

2. Possible Quantitative Forms for D-Value Variations

In a sterilization program using a probability distribution for D-values, denoted P(D) in the previous quarterly report, an enormous advantage accrues from knowing the quantitative form for P(D). Specifically, sampling programs or decisions to determine P(D) become sampling programs or decisions to determine

only the parameter values of P(D) - generally a much better defined and easier task.

Based on previous work it appears, at this time, that variations in thermal resistance are associated with the degree of stability of some substrate. This observation is based on a comparison of entropies and enthalpies of activation between B. subtilis var. niger spore inactivation and that of "hardy" spores from the soil at Cape Kennedy (see QR-21). If this is generally the case, then the variation in hardiness, P(D), stems from a variation in the activation entropy $\Delta S^{\neq}$, and the variation in hardiness may be described by a probability distribution of the form

$$\Pr\{\Delta S^{\neq} = -x\}, \tag{3}$$

since the rate of inactivation of spores in a thermal environment, k_T, may generally be represented in the form

$$k_T = \frac{KT}{h} \cdot e^{\Delta S^{\neq}/R} \cdot e^{-\Delta H^{\neq}/RT} = f_0(T)\, e^{\Delta S^{\neq}/R} . \tag{4}$$

Thus a knowledge of (3) allows the determination of the probability distribution of the rate of inactivation from (4) since

$$\Pr\{k_T = \alpha\} = \Pr\{f_o(T)\ e^{\Delta S^{\neq}/R} = \alpha\} = \Pr\{\Delta S^{\neq} = R \log\left(\frac{\alpha}{f_o(T)}\right)\}\ ,$$

and this latter is simply a specific form of (3).

The problem of determining a qualitative form for the variation in k_T among a population of organisms reduces, then, to the problem of deriving a distribution governing the activation entropy $\Delta S^{\neq}$ (making the above assumptions). Qualitatively, $\Delta S^{\neq}$ is a measure of the "stability" of the configuration of the reactants, and a number of possibilities for a more direct physical interpretation present themselves. For example, in a reaction type of the form

$$A \xrightarrow{k_T} \underline{\bar{X}} \qquad (5)$$

which inactivates a molecular complex A in the spore, one may imagine many modes by which A reaches state $\underline{\bar{X}}$. For example, if $\underline{\bar{X}}$ represents the generic state "denatured," then there are numerous ways that this may happen to A: Each chemically similar to any other. Let us suppose that there are M such modes of inactivation, and assume that each is equally likely - occurring with probability p_m. Then the probability that any s modes of inactivation are available at a given time might be represented by a binomial distribution

$$\Pr\{\text{modes} = s\} = \binom{M}{s} p_m^s\ (1 - p_m)^{M - s}\ . \qquad (6)$$

But if a molecule is very stable, it is easy to imagine that fewer modes of inactivation are available than if it is very unstable. Hence there would appear to be a relationship between the value of $\Delta S^{\neq}$ and (6), at least qualitatively. We may attempt to formalize this relationship by assuming first that (6) may be fairly approximated by a normal distribution (assuming M is large), that is

$$\Pr\{\text{modes} = x\} = \frac{1}{\sqrt{2\pi}\,\sigma}\, e^{-(x - \mu)^2/2\sigma^2} . \qquad (7)$$

Assuming then that $\Delta S^{\neq}$ has the same form of distribution as the availability of inactivation modes, we may let

$$\Pr\{\Delta S^{\neq} = -x\} = \frac{1}{\sqrt{2\pi}\,\sigma_s}\, e^{-(x - \mu_s)^2/2\sigma_s^2} . \qquad (8)$$

Under the circumstances envisioned above, equations (4) and (8) imply that k_T would be essentially log normally distributed. Although tenuously derived, the existence of a rationale and the fact that it appears to agree in shape with available data on D-value distributions from spacecraft surfaces makes investigation of this particular distribution desirable.

Assuming the distribution in (8) is the correct one leads to

$$\Pr\{k_T = \alpha\} = \frac{1}{\sqrt{2\pi}\,\sigma_s}\, e^{-[R \ln (\alpha/f_o(T)) - \mu_s]^2/2\sigma_s^2} \qquad (9)$$

where, as earlier, $f_o(T) = \frac{KT}{h}\, e^{-\Delta H^{\neq}/RT}$.

Thus the expression for expected survivors E(n(t)) at time t in a thermal environment becomes

$$E(n(t)) = n(o) \int_{\alpha=0}^{\infty} e^{-k_T t} \Pr\{k_T = \alpha\}\, d\alpha$$

or, using (8),

$$E(n(t)) = \frac{n(o)}{\sqrt{2\pi}\,\sigma_s} \int_0^{\infty} e^{-k_T t}\, e^{-[R \ln (\alpha/f_o(T)) - \mu_s]^2/2\sigma_s^2} \qquad (10)$$

If one studies a survivor curve of a naturally occurring spore population, such as that reported in our previous quarterly report (QR-21, June 1971), by taking samples at times t_o, t_1, ..., t_m, it is possible to determine μ_s and σ_s of equation (10) by numerically solving

$$\min_{\mu_s,\sigma_s} \sum_{r=o}^{m} [\ln E(n(t_r)) - \ln \bar{x}_r]^2 = \delta \qquad (11)$$

where $\bar{x}_r$ is the mean sample value at time t_r.

Letting $\varepsilon = \delta/(m - p)$, (12)

where p is the number of independent parameters in the distribution, the value of ε gives a measure of how "good" the representation (10) is for the survivor data.

First, for a given set of survivor data, one may use ε to compare the effectiveness of distributions governing k_T at representing the data. Thus it may be used to compare the log normal representation given in equation (10) with other distributions. If the rationale for the choice of the log normal distribution is correct, then in general (to within experimental error) it should perform better than others. In one study, both the exponential distribution

$$\Pr\{k_T = \alpha\} = \frac{1}{\lambda} \; e^{-\lambda/\alpha}$$

and the Rayleigh distribution

$$\Pr\{k_T = \alpha\} = \frac{\beta}{\alpha} \; e^{-\beta/2\alpha^2}$$

which have shapes similar to that of the log normal were used for comparison purposes. Secondly, ε is a measure of the applicability of a representation such as (10) when it is used to represent several sets of data. In particular, by measuring "goodness of

fit" with numerous sets of data, limits upon the applicability of (10) may be deduced.

The table below shows how this goodness of fit parameter, ε, varies with two data sets and three distributions.

Distribution Assumed on k_T

Data Set	Log Normal	Exponential	Rayleigh
Vacuum Dust Bldg. AO	0.1884	1.6273	4.2599
Cape Soil (PHS prep.)	0.6235	8.6571	3.0862

Goodness of fit (ε, equation 11) values for various distributions and thermal survival data from several naturally occurring (spore) populations.

In the context of this table, the log normal distribution for k_T appears reasonable. One specific parameter of interest is the mean value of the rate parameter k_T, denoted $\hat{k}_T$, or its counterpart mean D-value, $\hat{D}_T = \frac{2.303}{\hat{k}_T}$. This may be obtained from μ_s and σ_s.

For example, in the best fit of the log normal distribution for survivors from the Building AO dust, the mean D-value for the spores was

$$\hat{D}_T = 10.4 \text{ minutes.}$$

This value appears to be comparable to the mean D-values which have been found in studies of populations of microbes taken from spacecraft surfaces.

Thus, we see that from the reasoning used in Section 1, a technique should be developed which will take into account the variation in D-values of microorganisms on a spacecraft surface. The rationale and numerical findings of Section 2 indicate that a log normal distribution might be an appropriate choice. If one does choose such a distribution, then the planetary quarantine constraints may be met even though this variation in D-value does exist. A closed form is desirable since well defined sampling programs could then be established to determine the values of the parameters on the basis of the actual environment in which the spacecraft is assembled.

Thermoradiation Experimentation

A. Description. The objective of this activity is to thoroughly investigate the sterilizing effects of combinations of heat and ionizing radiation and to assess the practicality of this process for spacecraft sterilization. Thermoradiation may offer the possibility of sterilization at temperatures less than 100°C at low dose rates of approximately 10 krad/hour. This is possible because of a synergistic effect in bacterial inactivation which has been observed when combinations of heat and gamma radiation are applied simultaneously. Should any spacecraft components prove to be heat sensitive at high temperatures, thermoradiation offers a potential means of overcoming reliability problems. In addition, it provides a very effective means of inactivating highly resistant naturally occurring spores.

B. Progress. Activities this quarter have been devoted primarily to (1) radiation inactivation of Bacillus subtilis var. niger spores in nitrogen at room temperature and at a number of different dose rates, (2) radiation inactivation of Cape Kennedy soil spores in nitrogen at room temperature, and (3) investigation of thermoradiation resistance of Cape Kennedy soil spores at temperatures below 125°C.

Three experiments were run on B. subtilis var. niger spores to determine the radiation inactivation rate in nitrogen at room temperature. This data is necessary to define the radiation component of thermoradiation for optimizing synergism in a nitrogen atmosphere. The erratic nature of data in previous N_2 experiments necessitated additional experiments at room temperature.

Previous N_2 experiments were performed in what was thought to be a reasonably stable ambient temperature in the Gamma Irradiation Facility cell. It was found that this temperature would vary as much as 12 degrees in a 24-hour period and this was thought to have some effect on the inactivation results. To limit this temperature variation, an insulated chamber was made to hold the samples and the nitrogen was preconditioned to 23°C by flowing it through a coil in a warm water temperature bath. The nitrogen was checked to assure that a H_2O level of less than 10 PPM was maintained during experiments. Samples were prepared by pipetting an ethanol suspension of B. subtilis var. niger spores onto 1" x 1" aluminum planchettes. After evaporation of the ethanol, samples were placed in a vacuum over Drierite for 15 hours prior to sterilization treatment. Samples were exposed at radiation dose rates of 30 krads/hour, 80 krads/hour and 600 krads/hour. Results of these experiments are shown in Figures 1 through 3. Previous experiments at corresponding rates in air are also shown to illustrate the increased dose requirement in N_2 as compared to air.

One experiment was run at room temperature in nitrogen to determine the change in radioresistance of Cape Kennedy soil spores when irradiated in N_2. The preparation of the sample was the same as the previous B. subtilis experiments. Results are shown in Figure 4 where a comparison is made between inactivation of Cape soil spores in air and in nitrogen.

A series of experiments was begun to investigate thermoradiation inactivation of Cape soil spores at temperatures between 125°C and 105°C. Hopefully, inactivation of spores in soil can be accomplished at temperatures less than 125°C. Dose rates, sample intervals and moisture conditions were held constant with only temperature as a variable. Results of the first two experiments of this series are shown in Figure 5. Little change is detected between 125°C and 120°C, making a lower temperature potential rather interesting.

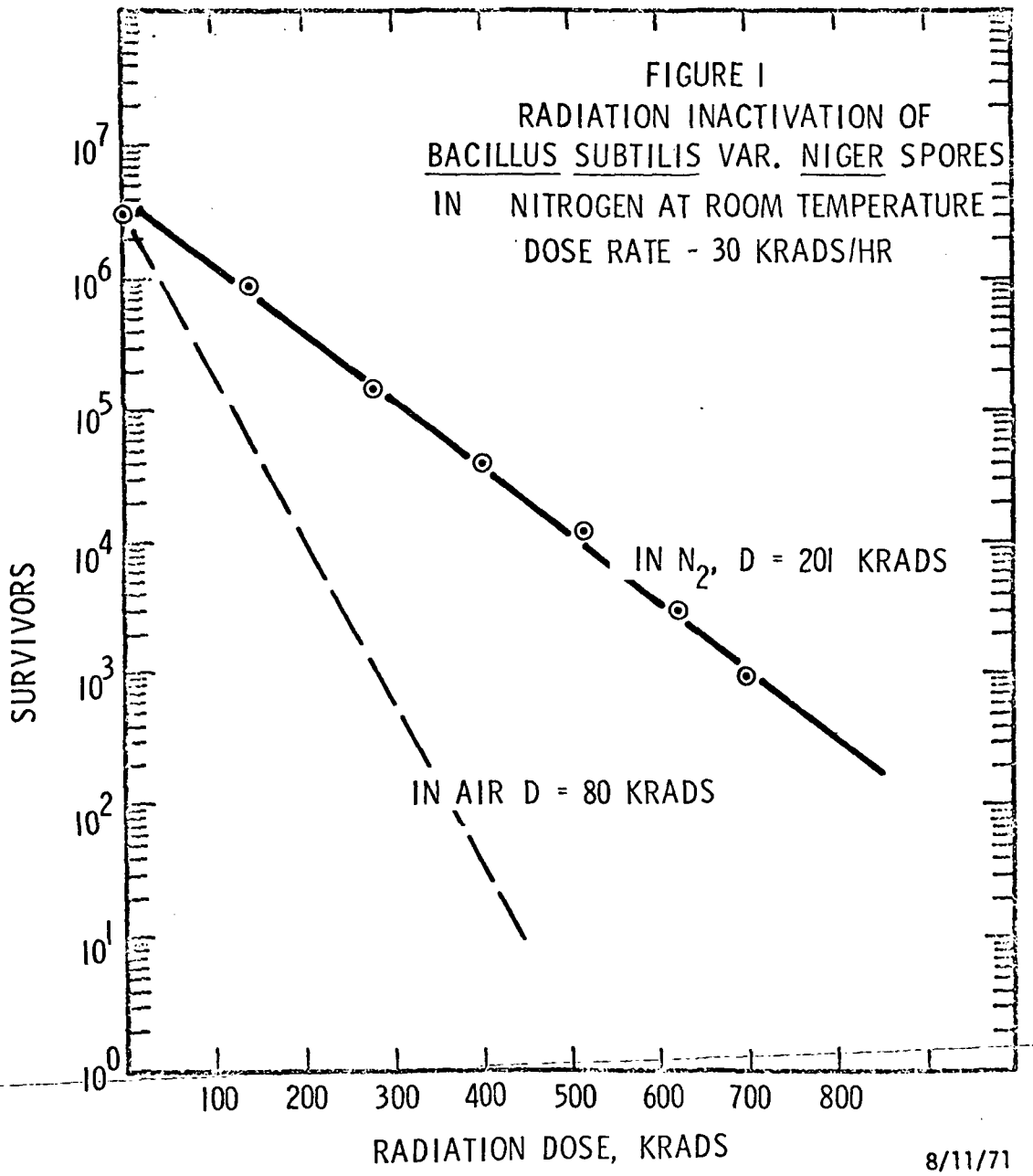


FIGURE I
RADIATION INACTIVATION OF
BACILLUS SUBTILIS VAR. NIGER SPORES
IN NITROGEN AT ROOM TEMPERATURE
DOSE RATE - 30 KRADS/HR
IN N_2, D = 201 KRADS
IN AIR D = 80 KRADS
SURVIVORS
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
100 200 300 400 500 600 700 800
RADIATION DOSE, KRADS
8/11/71

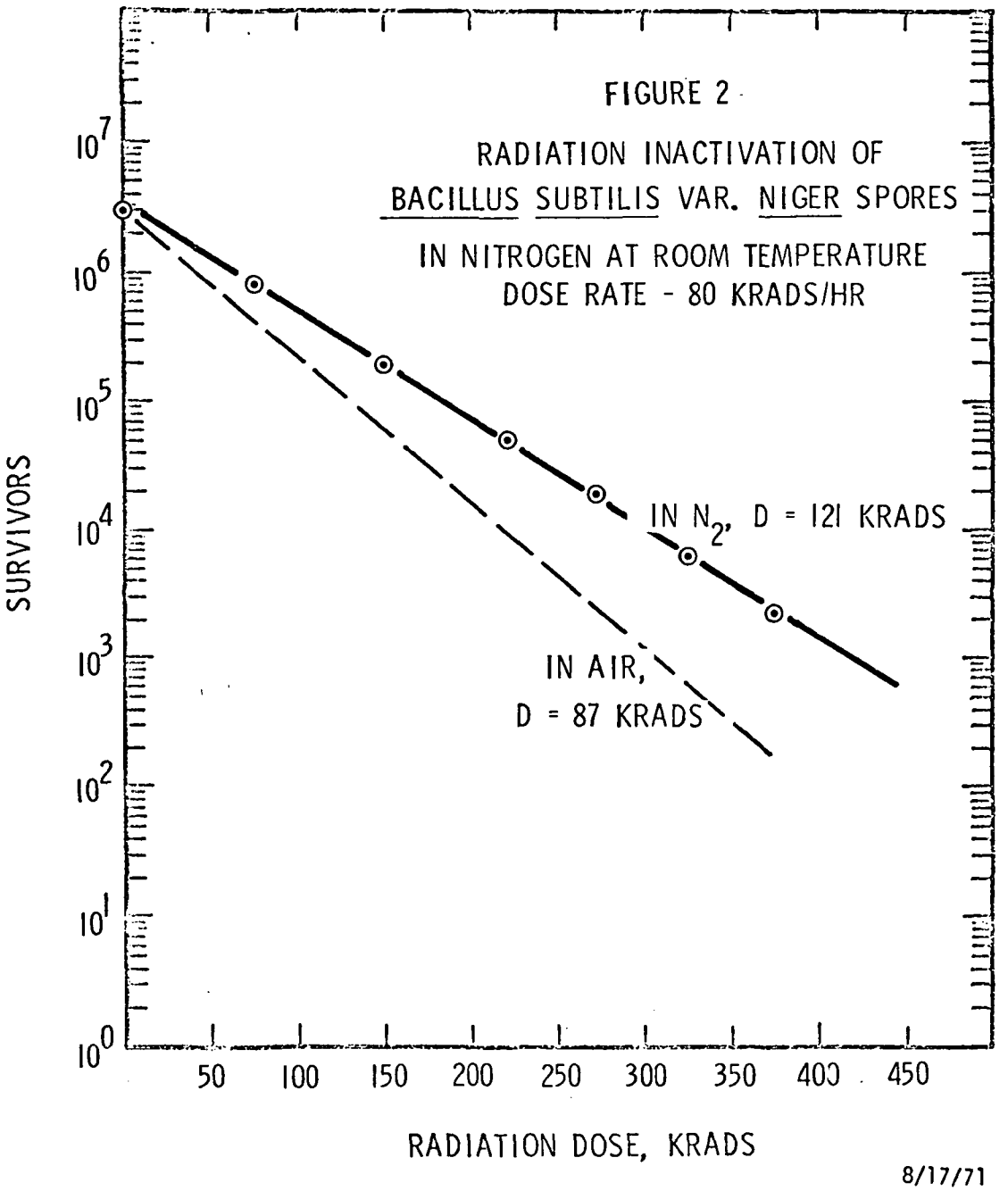


FIGURE 2
RADIATION INACTIVATION OF
BACILLUS SUBTILIS VAR. NIGER SPORES
IN NITROGEN AT ROOM TEMPERATURE
DOSE RATE - 80 KRADS/HR
IN N_2, D = 121 KRADS
IN AIR,
D = 87 KRADS
SURVIVORS
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
50
100
150
200
250
300
350
400
450
RADIATION DOSE, KRADS
8/17/71

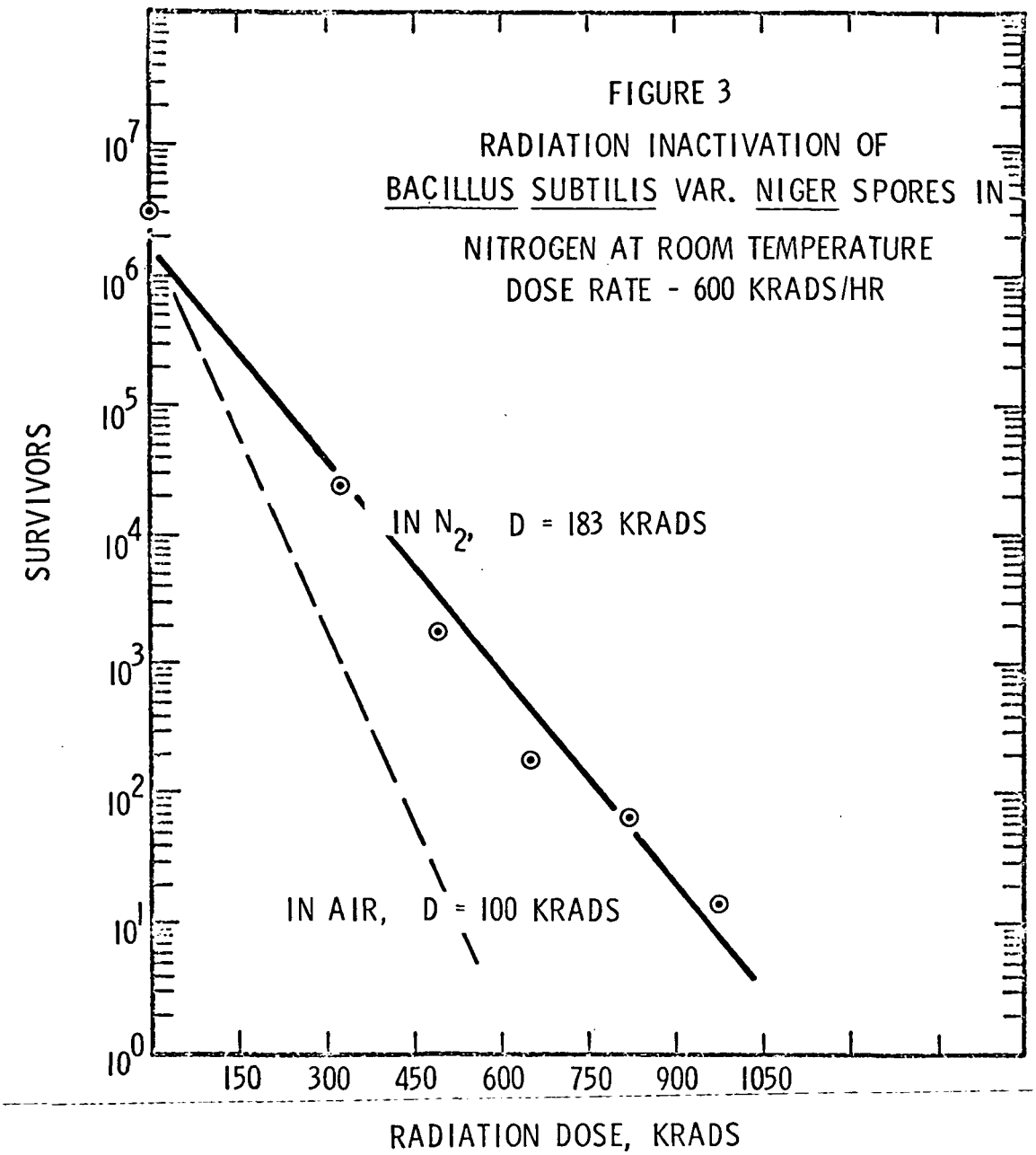


FIGURE 3
RADIATION INACTIVATION OF
BACILLUS SUBTILIS VAR. NIGER SPORES IN
NITROGEN AT ROOM TEMPERATURE
DOSE RATE - 600 KRADS/HR
IN N_2, D = 183 KRADS
IN AIR, D = 100 KRADS
SURVIVORS
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
150 300 450 600 750 900 1050
RADIATION DOSE, KRADS
8/18/71

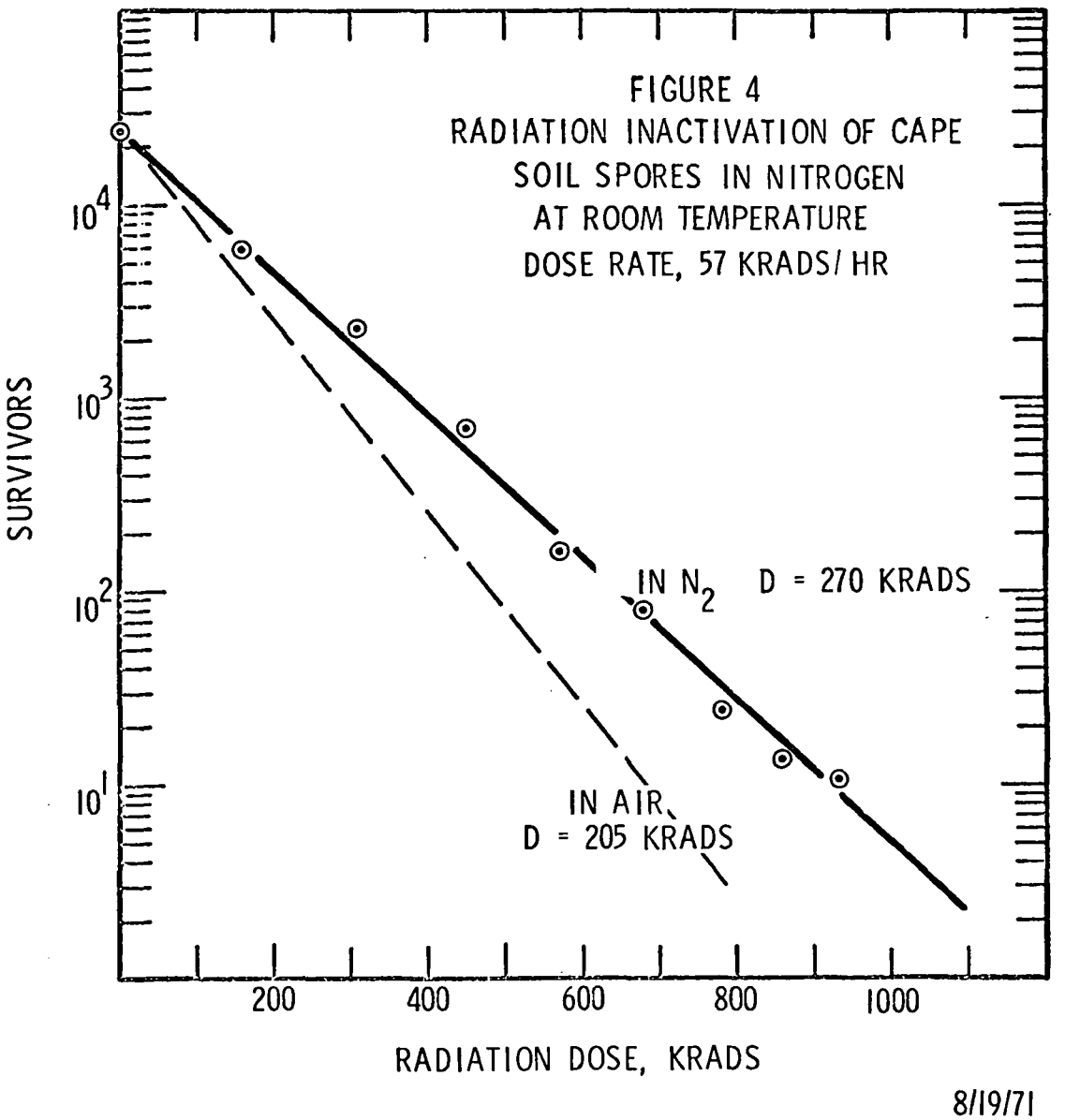
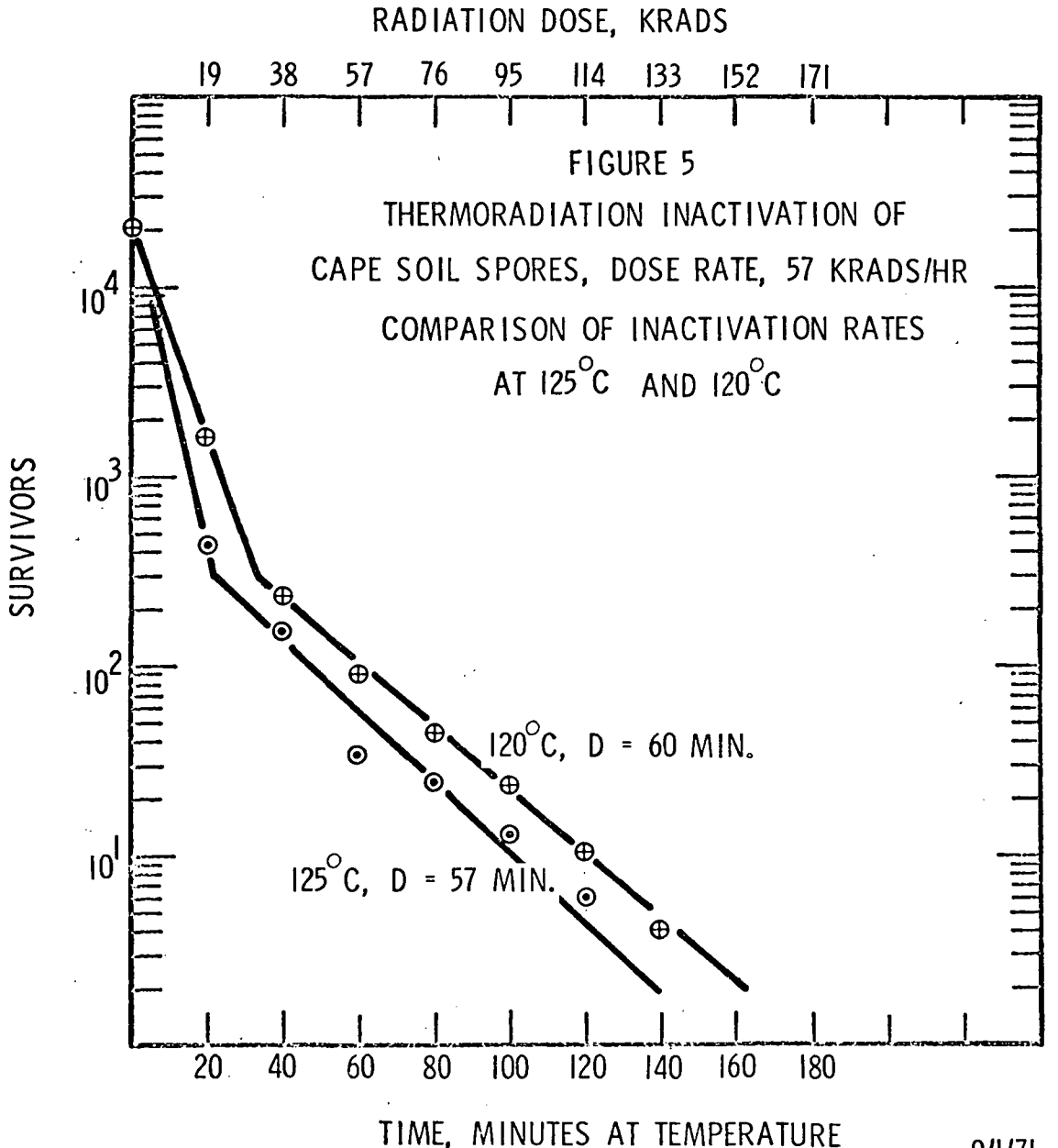


FIGURE 4
RADIATION INACTIVATION OF CAPE
SOIL SPORES IN NITROGEN
AT ROOM TEMPERATURE
DOSE RATE, 57 KRADS/ HR
SURVIVORS
10^4
10^3
10^2
10^1
IN N_2 D = 270 KRADS
IN AIR
D = 205 KRADS
200
400
600
800
1000
RADIATION DOSE, KRADS
8/19/71


RADIATION DOSE, KRADS
19 38 57 76 95 114 133 152 171
FIGURE 5
THERMORADIATION INACTIVATION OF
CAPE SOIL SPORES, DOSE RATE, 57 KRADS/HR
COMPARISON OF INACTIVATION RATES
AT 125°C AND 120°C
SURVIVORS
10^4
10^3
10^2
10^1
120°C, D = 60 MIN.
125°C, D = 57 MIN.
20 40 60 80 100 120 140 160 180
TIME, MINUTES AT TEMPERATURE
9/1/71

Study to Evaluate Possible Clumping Effects
Of Cape Soil On Highly Heat Resistant Spores

A. Description. Considerable interest has been aroused in the NASA Planetary Quarantine community by the high D-values exhibited by naturally occurring organisms found at Cape Kennedy. Dry heat D-values for these organisms have ranged from 8 to 30 hours at 125°C. Subcultures of the more heat resistant organisms from this source show D-values of up to 50 hours at 125°C. Since some difficulty has been experienced in completely separating the organisms from the soil particles, there was reason to suspect that the particles may be affording some degree of dry heat protection to the organisms during laboratory evaluation of the Cape soil. At the request of Dr. Larry Hall, a project was initiated to investigate this situation. In order to more nearly simulate spacecraft loading, it was suggested that these organisms be dispersed to a density of not more than 40 per square foot.

B. Progress. Several experiments were conducted to attain the desired microbial loading and to verify the results of exposure to a dry heat environment. These experiments are described below:

Materials and Methods. The stock used for these experiments was obtained by putting 5 grams of Cape Kennedy soil, < 147μ in size, through a series of Freon TF washing operations. An important feature of the stock preparation was the preconditioning of the dry soil particles prior to Freon washing. Due to the extreme hydrophobic quality of Freon and

the desire to work only with the more heat resistant organisms, the soil sample was heated in an evacuated oven for 1.5 hours at 125°C. At the end of this period, the oven was back-filled with dry nitrogen (N_2) prior to opening the door. The lightly capped container was then closed tight, removed from the oven, and allowed to cool to room temperature. Freon was added immediately upon removal of the cap. Thus ambient moisture was not permitted to contact the soil before the addition of Freon.

Each wash operation consisted of adding about 30 ml of Freon to the dust, insonating the mixture for 30 seconds, allowing the soil particles to settle for 30 seconds and drawing off the supernatant into a sterile beaker. This procedure was repeated 8 times. The collected supernatant was then subject to another series of similar washing operations. The supernatant from the second series of washes was filtered through a 0.8μ filter and the filter was insonated for two minutes in 50 ml of ethanol. This mixture constituted our base working stock of about 300 organisms per ml.

A new stock was prepared by diluting an appropriate amount of the original stock with ethanol. Our target was about 8 organisms per ml. One ml of the new stock was pipetted into each 150 mm glass Petri dish, which represents an area of ~0.2 square foot. The plates were rotated in such a manner that the solution covered the entire surface. After the excess ethanol had evaporated, the plates were placed in a recirculating oven at 125°C, and removed at hourly intervals up to 8 hours. Each hourly sample consisted of three plates which represented

about 3/5 of a square foot. After cooling to room temperature, the plates were overlayed twice with Trypticase Soy Agar with 0.1% soluble starch and 0.2% yeast extract added. The plates were incubated at 32°C and counted after 10 days incubation.

Results. Even with what appeared to be appropriate dilution of the original stock, we found it very difficult to attain a precise population of 40 organisms per square foot. In this experiment, we had an average of 16 organisms per square foot, which is probably a more realistic loading of heat resistent organisms on spacecraft surfaces. While this presented the situation of working with low numbers of colonies per plate, it also reduced the possibility of affording clumping protection to the organisms. The density and size of soil particles present on the plates are shown in Figures 1 and 2. Figure 1 represents a heavy loading for this experiment and Figure 2 a light loading. Each division on the attached scales represents 10μ. Further, the consistency of the data, especially when such low numbers are involved, suggests a high degree of validity (Figure 3).

The estimated D-value at 125°C was approximately 35-40 hours (Figure 4) in an extremely sparse population, where the likelihood of protection from clumping appeared to be negligible. This D-value compares favorably with D-values ascertained in other experiments where raw soil was used for experimental samples, for which the probability of clumping was many times greater.

Figure 1

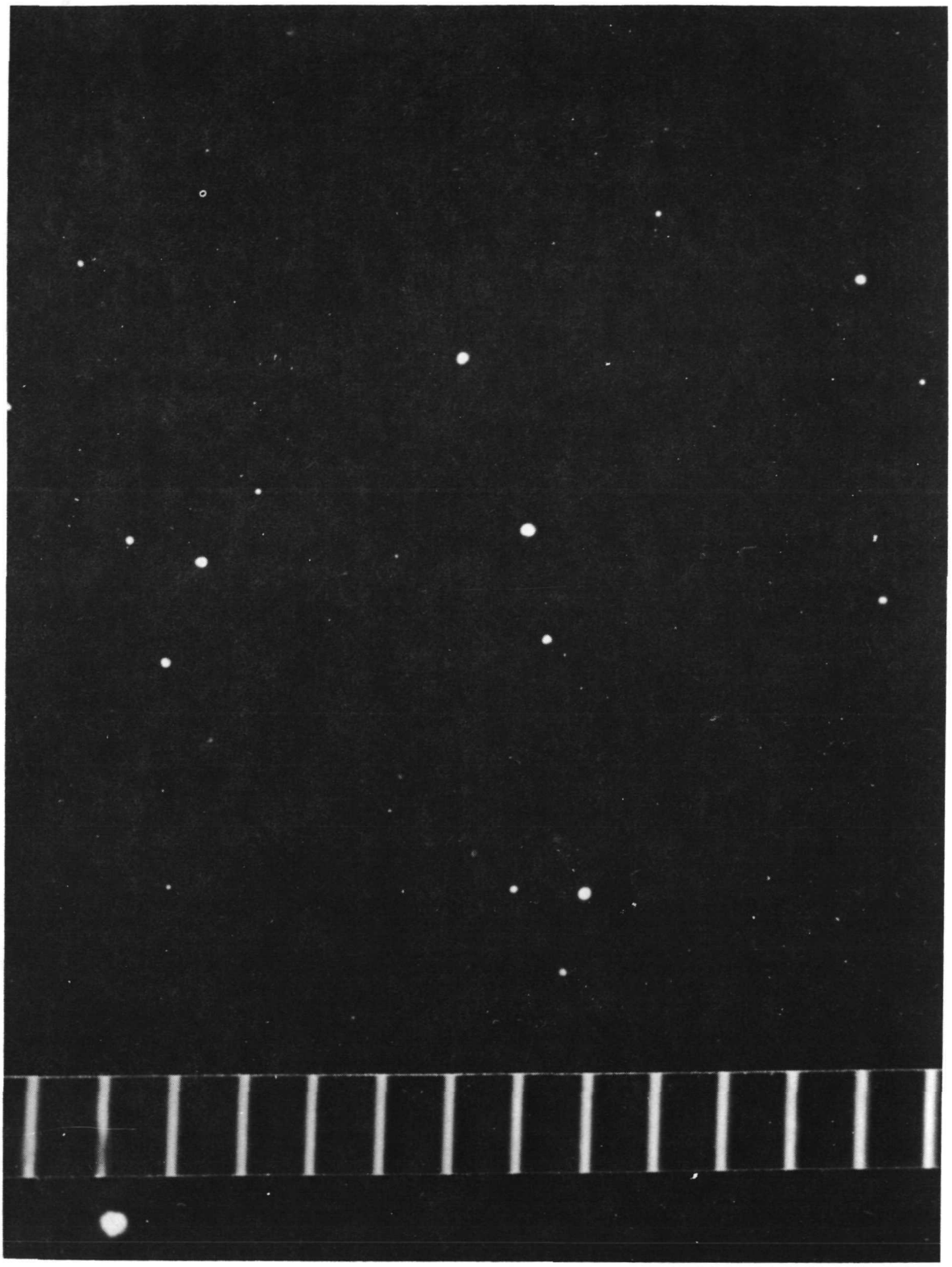


Figure 2

RAW DATA FOR EXPOSED PLATES								CONTROL PLATES	
HOURS AT 125° C	SAMPLE	PLATE COUNT	AVERAGE	HOURS AT 125° C	SAMPLE	PLATE COUNT	AVERAGE	PLATE NO.	PLATE COUNT
1	A	1	1.0	5	A	1	1.3	1	0
	B	0			B	2		2	2
	C	2			C	1		3	5
2	A	2	1.3	6	A	0	0.7	4	4
	B	0			B	2		5	4
	C	2			C	0		6	4
3	A	3	2.0	7	A	0	0.7		
	B	1			B	2		AVERAGE = 3.17	
	C	2			C	0			
4	A	1	1.0	8	A	0	0.0		
	B	1			B	0			
	C	1			C	0			

Figure 3

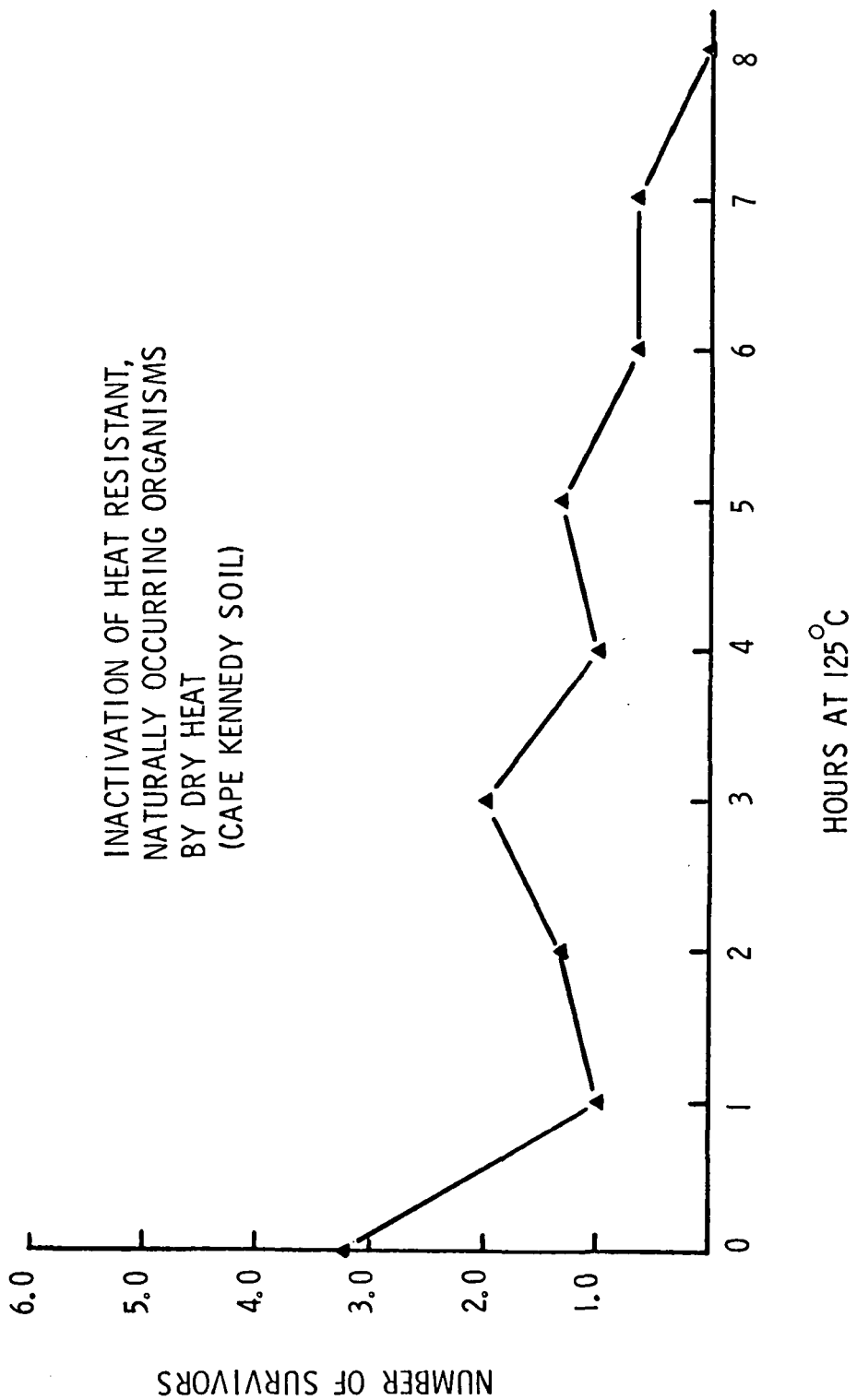

INACTIVATION OF HEAT RESISTANT,
NATURALLY OCCURRING ORGANISMS
BY DRY HEAT
(CAPE KENNEDY SOIL)
NUMBER OF SURVIVORS
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
HOURS AT 125°C

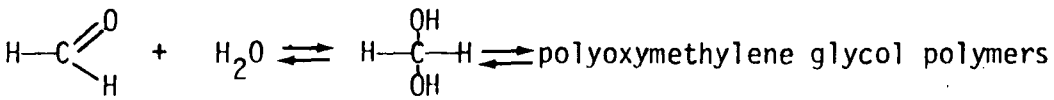
Figure 4

Studies on Bacterial Spore Inactivation

A. Description. A study demonstrating that the germination of bacterial spores can be inhibited by low concentrations of formaldehyde was described in QR-21. An interesting feature of this inhibition is that it is completely reversible. Spore germination proceeds once the spores are removed from a germinating media containing formaldehyde, via Millipore filtration or centrifugation, and resuspended in a germinating media free of formaldehyde. Thus, aqueous formaldehyde was shown to exert a sporostatic effect on spore development. However, the colony counts obtained from spore suspensions exposed to aqueous formaldehyde were nearly a log lower than the colony counts obtained from control spore suspensions containing the same initial spore concentration but not exposed to formaldehyde. This decrease in spore viability on exposure to aqueous formaldehyde indicates that aqueous formaldehyde can exhibit sporocidal properties in addition to sporostatic properties. This sporocidal property was investigated in more detail during this quarter's research activity.

B. Progress. The investigation into the effect of aqueous formaldehyde on the process of spore germination established the similarity of action of alcohols and formaldehyde on spore germination, i.e., both reversibly inhibit spore germination. Structurally and chemically, an aldehyde is different from an alcohol and yet both appeared to affect the spore germination process in a similar manner. Insight as to why formaldehyde should act as an alcohol in its effect on spore germination was obtained

from basic chemical considerations. Formaldehyde gas on dissolving in water rapidly reacts with water to form a monohydrate, methylene glycol, $CH_2(OH)_2$, and a series of low molecular weight polymeric polyoxymethylene glycols, having the type formula, $HO(CH_2O)_nH$. The reaction of formaldehyde with water is:



formaldehyde monomer — water — methylene glycol — polyoxymethylene glycol polymers

The methylene glycol combines with other molecules of methylene glycol to yield polymers of polymethylene glycol of varying molecular weight. It has been determined that at 30°C a 2% formaldehyde solution contains 0.001% formaldehyde monomer and 99.999% methylene glycol and polymers of methylene glycol. Formaldehyde in an aqueous medium is 99.999% in the form of a di-alcohol (methylene glycol) or polymeric di-alcohols. Therefore, formaldehyde can act like an alcohol in inhibiting spore germination because in an aqueous solution formaldehyde exists essentially as a di-alcohol. That di-alcohols *per se* can inhibit spore germination was shown in a study in which ethylene glycol reversibly inhibited the germination of *B. subtilis* var. *niger* spores.

This equilibrium can also account for the sporocidal property of aqueous formaldehyde if one assumes that the low level (0.001%) of formaldehyde present as the aldehyde in aqueous formaldehyde solution is the agent causing spore inactivation. Since the reaction of formaldehyde with water is an equilibrium system, then an alteration of the

equilibrium position of the reaction would be expected to alter the concentration of free formaldehyde present in an aqueous formaldehyde solution. Such a change in the concentration of free formaldehyde should then be manifest as a change in the rate of spore inactivation. Temperature is known to be a parameter which can influence the equilibrium position of a chemical reaction. Therefore, the effect of temperature on the sporocidal activity of an aqueous formaldehyde was studied. Figure 1 presents the results of this study. Heating B. subtilis var. niger spores in an aqueous suspension from 30-60°C for four hours resulted in no decrease in spore viability. Incubating B. subtilis spores in a 1% formaldehyde solution at room temperature (23°C) resulted in approximately a one log decrease in spore survival levels after a four hour exposure. Increasing the incubation temperature from 30°C through 60°C for B. subtilis spores suspended in a 1% aqueous formaldehyde solution resulted in dramatic changes in the rate of spore inactivation. For example, the test samples run at 55°C and 60°C for four hours were completely inactivated. Sterility was determined by direct colony count and by incubation in broth for ten days. Dilution bottles containing either 0.5% sodium sulfite or ammonium chloride were used to serially dilute spore suspensions in two different experiments in an effort to neutralize the formaldehyde and, perhaps, reverse the formaldehyde induced inactivation. No difference in survival level was observed between these experiments and those in which formaldehyde treated spores were not exposed to these chemicals.

The equilibrium between formaldehyde monomer and its di-ol form in water (methylene glycol) is not greatly affected by changes in temperature up to 60°C. There is approximately a ten fold increase in the free formaldehyde concentration at 60°C compared to 30°C. Therefore, the extreme temperature dependence observed for the inactivation of spores in aqueous formaldehyde may be a result of the increased rate of reaction between free formaldehyde monomer and the spores with increasing temperature. Consistent with such an interpretation is the observation that spores suspended in 1% formaldehyde solution and heated to 50°C, 55°C, or 60°C all exhibited approximately the same survival level after treatment for thirty minutes and thereafter had different rates of inactivation (Figure 1). This result can be understood if one assumes that sufficient thermal energy was present at 50°C - 60°C to allow the equilibrium concentration of formaldehyde monomer to be completely utilized in combining with and inactivating a certain fraction of the total spore population. The subsequent differences in spore inactivation rate from 50°C - 60°C could then reflect the influence of temperature on the re-establishment of the equilibrium between formaldehyde monomer and methylene glycol in an aqueous formaldehyde solution.

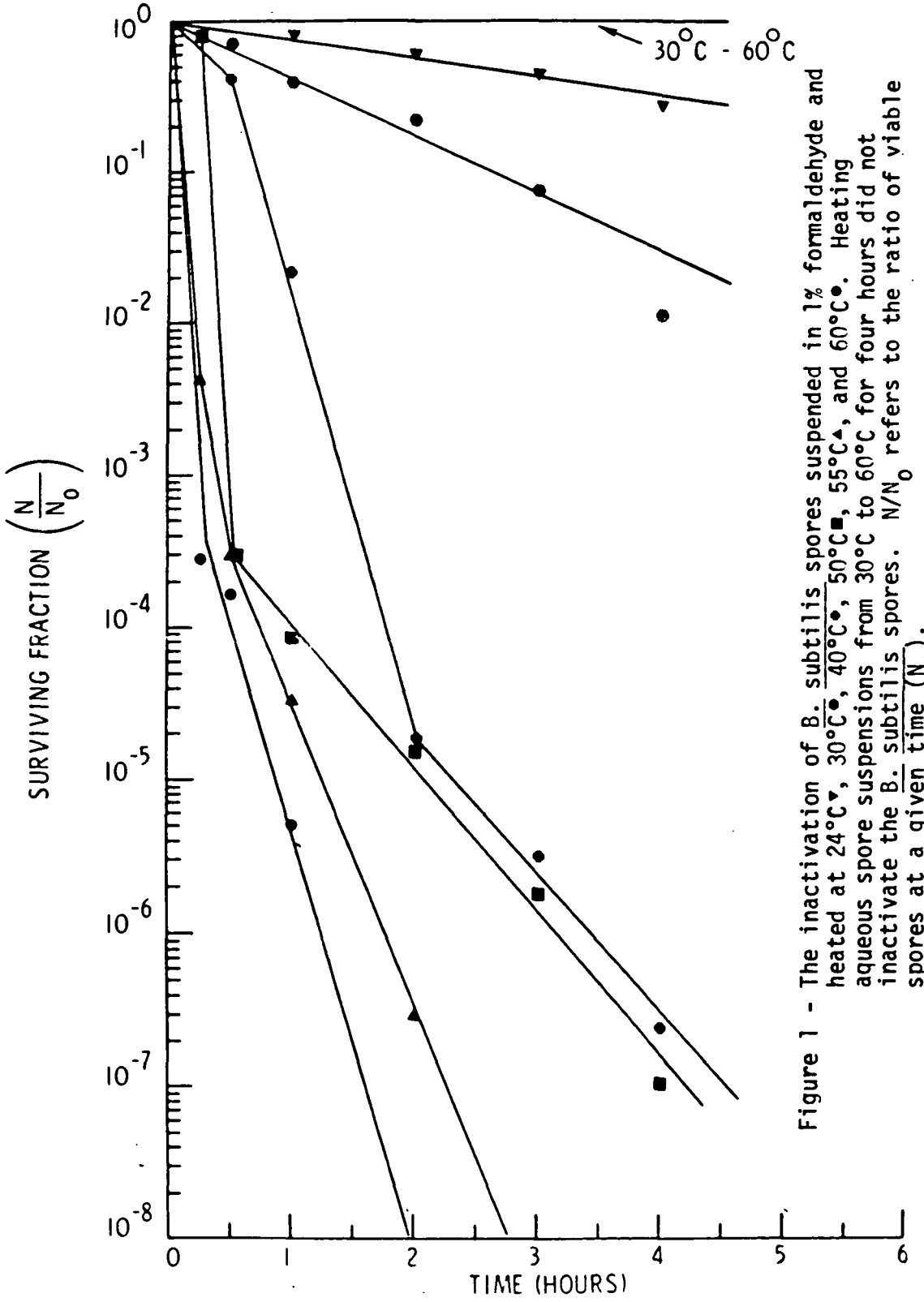


Figure 1 - The inactivation of B. subtilis spores suspended in 1% formaldehyde and heated at 24°C▼, 30°C●, 40°C●, 50°C■, 55°C▲, and 60°C●. Heating aqueous spore suspensions from 30°C to 60°C for four hours did not inactivate the B. subtilis spores. N/N_0 refers to the ratio of viable spores at a given time (N_0).

Federal Standard 209a

A. Description. Sandia Laboratories, through the AEC, has the delegated responsibility from the General Services Administration (GSA) to maintain Federal Standard 209a, "Clean Room and Work Station Requirements, Controlled Environment."

B. Progress. By letter from the Sandia Area Office, USAEC, Sandia Laboratories has been officially designated by the GSA as the preparing activity for Revision B to Federal Standard 209a. While minimal maintenance activity has been carried on in the past, this letter constitutes authorization to proceed with a formal revision of the Standard. As an initial step, letters have been prepared requesting the following agencies to appoint their representative for this project:

National Aeronautics and Space Administration
Atomic Energy Commission
Department of Defense
Department of the Air Force
Department of the Navy
Department of the Army

Other agencies may be added to this list if we determine later that they have sufficient interest in the Standard to warrant participation in the revision.

Publications

1. J. P. Brannen, "Role of Water Activity in the Dry Heat Sterilization of Microorganisms," Journal of Theoretical Biology, Vol. 32, No. 2, pp. 331-334, August, 1971.
2. R. E. Trujillo, "Small Concentrations of Octanol Inhibit Bacterial Spore Growth," appeared in Sandia Science News, Vol. 6, No. 3, September 1971.
3. R. T. Dillon (1741), Diane Holdridge (5162) and R. Puleo and G. Oxborrow, PHS, Cape Kennedy, Florida, "A Computerized Bacterial Identification System as Applied to Planetary Quarantine," Space Life Sciences, Vol. 3, No. 1, pp. 63-84, August 1971.
4. R. E. Trujillo and V. L. Dugan, "Analysis of the Synergistic Inactivation of Viruses by Thermoradiation," Biophysical Journal, to appear.
5. "Contamination Control - A State-of-the-Art Review," Proceedings of the First European Symposium, Stuttgart, West Germany, W. J. Whitfield and D. M. Garst.
6. D. M. Garst, "The Role of Laminar Airflow in Hospitals: A Case History," Contamination Control, September-October, 1971, 10:13-17.
7. D. M. Garst, "Communication," Guest columnist for the president, AACC, Contamination Control, September-October, 1971, 10:4.

Presentations and Briefings

1. H. D. Sivinski spoke to the New Mexico Society of Hospital Pharmacists, July 7, on "Planetary Quarantine."
2. H. D. Sivinski assisted Dr. George Fishbeck in presenting the TV science program for September 20 on KNME for the sixth grade science classes of the Albuquerque Public School System on "Planetary Quarantine."

Meetings

1. D. M. Garst participated in a meeting of the Executive Committee of the American Association for Contamination Control in Raleigh, North Carolina on September 19-20, 1971.

Consultants Who Visited During Quarter

1. Dr. John Brewer - Hardin-Simmons University, Brownwood, Texas.
2. Dr. H. O. Halvorson - University of Minnesota, St. Paul, Minnesota.
3. Dr. Henry Eyring - University of Utah, Salt Lake City, Utah.

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